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A major genetic event that occurs in the pathogenesis of breast carcinoma involves alterations in the Bcl-2 survival pathway. To determine the role of the Bcl-2 family in maintaining cancer cell viability, we constructed a recombinant adenovirus vector that expresses Bcl-x$_S$, a dominant inhibitor of these proteins. In the original proposal, we proposed studies to determine the mechanism involved in Bcl-x$_s$-mediated apoptosis, to characterize cellular proteins that interact with Bcl-x$_S$ using biochemical and genetic approaches, and to use a transgenic model of Bcl-x$_S$ expression in the breast to assess the requirement for Bcl-2/Bcl-x$_L$ in the maintenance of normal breast epithelia and tumor growth. During the last two years, we have characterized the interaction of Bcl-x$_s$ with Bcl-2 and Bcl-x$_L$ and identified through a genetic screen, HRK, a novel protein that interacts with Bcl-2 and Bcl-x$_L$. Mutational analysis of Bcl-x$_s$ revealed a conserved region of this protein that is important for interaction with Bcl-x$_L$ and induction of apoptosis. Bcl-x$_L$ and Bcl-x$_s$ were found to associate with Apaf-1, a critical regulator of caspases and cell death, suggesting that Bcl-x$_s$ might antagonize Bcl-x$_L$ through Apaf-1. Finally, transgenic mice expressing Bcl-x$_s$ in the breast have been developed and partially characterized.

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FOREWORD

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INTRODUCTION

Cancer is the result of multiple genetic events, including activation of oncogenes and inactivation of tumor suppressor genes. The protein products of the former are often mitogens, whereas the products of the latter suppress proliferation. It is becoming increasingly apparent that tumor suppressor genes like p53 function in part by activating an apoptotic death pathway. In addition, certain oncogenes such as bcl-2 appear to contribute to tumor development primarily by promoting abnormal cell survival via an apoptosis inhibitory signal. Thus, disruption of the apoptosis pathway appears integral to many malignancies including breast cancer. Furthermore, treatment of cancer with chemotherapy or radiation therapy is limited by the emergence of tumor cells resistant to these therapies. This resistance limits our ability to successfully treat these neoplasms.

bcl-2, the first member of an evolutionarily conserved family of apoptosis regulatory genes, was initially isolated from the t(14; 18) chromosomal translocation found in human B-cell follicular lymphomas, and was subsequently shown to repress cell death triggered by a diverse array of stimuli (1-2). Several members of the family, including Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1/Bfl-1, share conserved regions termed Bcl-2 homology domain 1, 2, 3, and 4 (BH1, BH2, BH3, and BH4), and function by repressing apoptosis (1-4). In contrast, structurally related proteins, including Bax, Bak, Bad, Bik/Nbk, Bid, Hrk, Bim, and Bok/Mtd, activate apoptosis (5-11). Biochemical and functional analyses have revealed that these pro-apoptotic proteins require the conserved BH3 region to interact with Bcl-2/ Bcl-x_L, and activate apoptosis in transient assays (5-11). Moreover, NMR studies have demonstrated that the BH3 domain of Bak interacts with a hydrophobic cleft formed by the conserved BH3 and BH1 regions of Bcl-x_L (12). To date, all death-promoting Bcl-2-related proteins heterodimerize with Bcl-2, Bcl-x_L, or Mcl-1, suggesting that these molecules promote cell death at least in part by interacting with and antagonizing Bcl-2, Bcl-x_L, and Mcl-1 (5-11). The biochemical process by which pro-survival Bcl-2 family members regulate cell death is poorly understood. It has been proposed that pro-survival family members regulate apoptosis by maintaining the integrity of the mitochondria (13). In addition, these Bcl-2 family proteins have been proposed to regulate apoptosis via physical interactions with central caspases through adaptor molecules, such as Apaf-1 and CED-4 (14-15).

A major genetic event that occurs in the genesis and/or progression of breast carcinoma involves alterations in the pathway of apoptosis. In breast cancer, one of the most common abnormalities is deregulated expression of Bcl-2 or Bcl-x_L proteins. Up to 90% of cancers originating from breast overexpress Bcl-2 or Bcl-x_L (16-17). We hypothesize deregulated expression of these proteins plays a critical role in the maintenance of breast cancer cells and resistance of tumor cells to therapy-induced apoptosis. To determine the role of the Bcl-2 family of proteins in maintaining cancer cell viability, we constructed a recombinant adenovirus vector that expresses Bcl-x_S, a dominant inhibitor of Bcl-2 and Bcl-x_L. Even in the absence of an exogenous apoptotic signal, this recombinant virus specifically and efficiently activates apoptosis in human carcinoma cells arising from multiple organs including breast, colon, stomach and sympathetic nervous tissue (18). Based on these results, we hypothesize that apoptotic signals are constitutively expressed in proliferating cancer cells and perhaps in normal cells, although repressed by members of the Bcl-2 family of proteins. In this proposal, we proposed studies (i) to determine the mechanism involved in Bcl-x_S-mediated apoptosis using the *bcl-x_S* adenovirus

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to dissect molecular interactions of the Bcl-2 regulatory pathway; and (ii) to characterize cellular proteins that interact with Bcl-x$_S$ using biochemical and genetic approaches. The studies outlined in this proposal may provide novel insight into the apoptosis pathway and lead to alternative therapeutic strategies for the treatment of breast cancer and other malignancies.

BODY OF THE ANNUAL REPORT

Technical Objective #1: Further characterization of the interaction of Bcl-x$_S$ with Bcl-2, Bcl-x$_L$ and Bax in breast cancer cells.

Task 1.1: Determine whether Bcl-x$_S$ associates with Bcl-x$_L$ and/or Bcl-2 *in vivo*.

We presented evidence in the original proposal that MCF-7 breast tumor cells and primary breast carcinoma cells undergo apoptotic cell death after exposure to a *bcl-x$_S$* adenovirus vector. Our hypothesis was that inactivation of Bcl-2 or Bcl-x$_L$ by Bcl-x$_S$ unleashes endogenous death signals leading to execution of the apoptotic program. We proposed experiments to examine molecular interactions of the Bcl-2 family members following expression of Bcl-x$_S$ protein, but prior to morphological/biochemical cell death, in order to define those interactions which may be created or destroyed as the apoptotic program is activated.

Although these experiments were originally done in *bcl-x$_S$* adenovirus infected cells, we have since used transiently transfected cancer cells for the characterization of protein-protein interactions. Doing so has allowed us to perform immunoprecipitaions of epitope tagged Bcl-x$_S$, which were previously impossible with the untagged *bcl-x$_S$* adenovirus. This model system is similar to that of the *bcl-x$_S$* adenovirus in that cancer cells undergo apoptosis in response to transient transfection of both untagged and HA-epitope tagged Bcl-x$_S$. To determine if Bcl-x$_S$ associates with Bcl-x$_L$ or Bcl-2, sequential Immunoprecipitation/Western–blot analysis was done on lysates of cells transfected with Bcl-x$_S$-HA and Bcl-x$_L$-Flag or Bcl-2-Flag. Lysates were immunoprecipitated with either anti-Flag or anti-HA antibodies. Washed protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with the reciprocal antibody. These experiments showed that Bcl-x$_S$ interacts with both Bcl-x$_L$-Flag and Bcl-2 Flag, although perhaps more weakly than with Bcl-x$_L$ (Fig. 1.)

In additional experiments, we have performed extensive mutagenesis of the Bcl-x$_S$ protein to determine the regions of Bcl-x$_S$ required for its interaction with Bcl-x$_L$ and those involved in breast cancer cell killing. We have expressed the Bcl-x$_S$ mutants in cells *in vivo* (Fig. 2a), and have analyzed these mutants for their ability to kill a variety of cancer cells in the transient transfection assay. Results of these studies have mapped the domain required for killing as the BH3 domain (amino acids 86-98) (Fig. 2b.) Interestingly, the broad range caspase inhibitor, viral p35, is able to completely inhibit Bcl-x$_S$ killing, suggesting that Bcl-x$_S$ kills through a caspase dependent mechanism. We are currently analyzing these mutants for their ability to bind Bcl-x$_L$, both *in vitro* in the yeast 2-hybrid system, and *in vivo* in transiently transfected cancer cells.

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<u>Task 1.2</u>: Determine whether Bcl-x$_S$ disrupts the interaction of Bcl-x$_L$ with Bax and Bak

We determined next if Bcl-x$_S$ expression could alter the interaction of Bcl-x$_L$ with BAX in transiently transfected cancer cells. In these experiments, FLAG-tagged Bcl-x$_L$/Bax complexes were immunoprecipitated with anti-FLAG in the presence or absence of Bcl-x$_S$, and the amount of bound endogenous BAX was determined by immunoblotting with anti-BAX antibody. The results showed that Bcl-x$_S$ is unable to displace BAX from Bcl-x$_L$, even in cells that undergo Bcl-x$_S$ mediated cell death (Fig. 3.) We could not determine if Bcl-x$_S$ could alter Bcl-x$_L$ binding to BAK, because the cancer cell lines we tested did not express detectable levels of endogenous BAK.

<u>Task 1.3</u>: Determine whether Bcl-XS fucntions by association with other proteins that regulate apoptosis

Recent experiments with nematode CED-9, CED-4 and CED-3 have suggested that Bcl-2 and Bcl-x$_L$ regulate apoptosis by interacting with and inhibiting caspases (death proteases) through Apaf-1, a mammalian homologue of the *C. Elegans* CED-4 protein (14-15). These observations suggested that Bcl-x$_S$ might antagonize Bcl-x$_L$ by interfering with the ability of Bcl-x$_L$ to inactivate the caspase regulator Apaf-1. We performed experiments to test whether Bcl-x$_L$ could associate with Apaf-1 or procaspase-9, a central death protease that associates with Apaf-1 (19). The completion of these experiments with Bcl-x$_L$ was important prior to the analysis of Bcl-x$_S$ function. The analysis showed that Bcl-x$_L$ and caspase-9 co-immunoprecipitated *in vivo* (Fig. 4a). As the nematode CED-9 protein, a homolog of mammalian Bcl-x$_L$, interacts with CED-3 through CED-4 (20-23), we determined if Bcl-x$_L$ could associate with Apaf-1. Immunoblotting of Bcl-x$_L$ complexes with anti-Myc antibody revealed that Apaf-1 co-immunoprecipitated with Bcl-x$_L$ (Fig.4b). To verify these results, we performed reciprocal experiments in which wt Apaf-1 and Apaf-1 deletion mutants were immunoprecipitated with anti-Myc antibody. Immunoblotting with anti-Flag confirmed that Bcl-x$_L$ co-immunoprecipitated with Apaf-1 and revealed that Bcl-x$_L$ associates with both the CED-4-like domain and the C-terminal region that contains the WD repeats of Apaf-1 (Fig. 4c).

These observations suggest that Bcl-2/Bcl-x$_L$ control apoptosis at least in part through physical association with Apaf-1, and that Bcl-x$_S$ might promote apoptosis by interfering with the ability of Bcl-x$_L$ to associate with Apaf-1. We tested first whether Bcl-x$_S$, was capable of interacting with Apaf-1. The analysis showed that Bcl-x$_S$ co-immunoprecitated with Apaf-1 (Fig. 5). This result indicates that regions outside the BH1-2 domains that are present in Bcl-x$_S$ may mediate contact with Apaf-1. These preliminary results suggest that Bcl-x$_S$ may promote apoptosis by competing with Bcl-x$_L$ for Apaf-1 binding. Alternatively, the cellular interaction we have observed between Apaf-1 and Bcl-x$_S$ might be indirect and mediated by binding of Bcl-x$_S$ to endogenous Bcl-x$_L$ or another adaptor protein. Future experiments to be conducted in the coming year will be performed to discriminate between these two models. These will include yeast two-hybrid analysis of the Bcl-x$_S$ association with Apaf, and use of the Bcl-x$_S$ deletion mutants to map region(s) required for this interaction.

<u>Technical Objective #2: Further characterization and purification of p15, a cellular protein that interacts with Bcl-x$_S$.</u>

A mechanism that might explain the apoptosis-promoting activity of Bcl-x$_S$ is through binding to an upstream activator or a downstream effector of Bcl-2/Bcl-x$_L$ mediated survival. In

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preliminary results, we provided evidence in the original application that Bcl-x$_S$ interacts with a cellular protein, p15, in cancer cells infected with the *bcl-x$_S$* adenovirus. The significance of the Bcl-x$_S$-p15 interaction was unclear. p15 could represent a death effector which is activated by the expression of Bcl-x$_S$. Alternatively, p15 could be a cellular protein required for survival whose activity is inhibited by the Bcl-x$_S$ interaction. Clearly, biochemical characterization of p15 and isolation of the p15 cDNA was needed to further assess the role of p15 in Bcl-x$_S$-mediated apoptosis.

Task 2.1: Biochemical Purification and N-Terminal Microsequencing of p15

We purified p15 by virtue of its association not only with Bcl-x$_S$, but also with Bcl-2. Stably transfected Shep-1 nueroblastoma cells expressing Bcl-2 Flag were used as a source of the Bcl-2-associated p15 protein. We purified p15 isolated from preparative gels and submitted the material for microsequencing. The N-terminal sequence we obtained from the p15 material was: M/K-R-D-P-V-A-R-T-S. Except for the first amino acid, this sequence corresponds to amino acids of human Bcl-2 that are part of its flexible loop region (24). Two potential caspase cleavage sites occur within the loop at positions 34 and 64. However, the Bcl-2 fragment we sequenced failed to corresponded to either caspase cleavage site. It is most likely the result of non-specific protealysis of the exposed loop region, and is therefore a degradation product of Bcl-2. The original p15 protein identified as a Bcl-x$_S$ binding protein is clearly different from the p15 we observed as a Bcl-2 associated band. However, we have been unable to detect any 15 kDa protein associating with Bcl-x$_S$-HA in variety of cancer lines tested. As the original p15 was detected in adenovirus Bcl-x$_S$-infected cells, it is possible that the p15 was an adenoviral gene product. Given these results we are no longer pursuing the characterization of the protein band termed p15.

Task 2.2: Genetic Screen for Bcl-x-Binding Proteins using the Two-Hybrid Yeast Assay

To search for Bcl-x-interacting proteins, we screened placenta and brain cDNA libraries using Gal4-Bcl-x$_L$ and Gal4-Bcl-x$_S$ as "baits" in the yeast two-hybrid assay. In the first screen using the GAL4-Bcl-x$_L$ bait, fifty-six positive clones were identified that interacted with Gal4-Bcl-x$_L$. Forty-one cDNAs encoded Bad, a Bcl-2 family member recently isolated by binding to Bcl-2. Ten cDNAs encoded Bcl-2, which is known to bind Bcl-x$_L$ in the two-hybrid system. The nucleotide sequences of four cDNAs were novel in that they did not reveal significant homology to any known gene or translated products in the databases. Three of these novel cDNAs encoded the same gene, which we have named *harakiri*. The same gene was identified as an interacting partner of Bcl-2. *Harakiri* functions as a regulator of Bcl-2 and Bcl-x$_L$ and apoptotic cell death in mammalian cells. The *hrk* product (HRK) does not exhibit significant homology to Bcl-2 or Bcl-x$_L$ and lacks the conserved BH1 and BH2 domains that are shared by Bcl-2 family members. Significantly, HRK physically interacts with anti-apoptosis proteins Bcl-2 and Bcl-x$_L$ but not with death-promoting Bcl-2 members such as Bax and Bak. Expression of HRK induces rapid onset of cell death in mammalian cells including breast cancer cells. Importantly, the death-promoting activity of HRK is repressed by Bcl-2 and Bcl-x$_L$, suggesting that HRK is a common target of the anti-apoptosis proteins Bcl-2 and Bcl-x$_L$. Details of these results have been recently published (Inohara *et al.* The EMBO J. 16:1686-1694, 1997). Because analysis of HRK is beyond the statements of work, we are not pursuing the analysis of HRK with funds provided by this grant.

<u>Technical Objective #3: Analysis of Transgenic Mice Expressing Inducible Bcl-XS in the Breast.</u>

<u>Task 3.1</u>: Expression analysis of transgenes

Previous attempts in our lab to generate Bcl-x$_S$ transgenic mice under a variety of constitutively active promoters have failed. In an attempt to elucidate the *in vivo* function of Bcl-x$_S$, we generated Bcl-x$_S$ transgenic mice under the control of the WAP (Whey acidic protein) promoter, which is induced during lactation. .Potential Wap-Bcl-x$_S$-HA transgenic mice were first identified by PCR analysis using Bcl-x-specific promoters that flank an intron. Seventeen lines were identified as being positive by PCR analysis of genomic DNA. Of these, thirteen were screened by Western blotting of lactating mammary gland tissue at day L10 of lactation. Of these, two positive transgenic lines were identified by anti-HA or anti-Bcl-x Western blotting. An anti-Bcl-x western blot screening of the first group of mice is shown in Fig. 6a.

<u>Task 3.2</u>: Histological evaluation of mice
Of the two Bcl-x$_S$ HA-expressing lines identified, both were able to lactate normally as evidenced by the successful nursing of pups and normal H and E staining (Fig. 6b and c.) However, in both lines, females failed to lactate during the second pregnancy (Fig. 6d.) We are currently performing experiments to determine why this occurs. One possibility is that they truly fail to lactate, another is that they might undergo premature involution. Future experiments will involve harvesting mammary tissue at various time points throughout the first and second pregnancies. Tissue samples will be analyzed by anti-HA Western blotting and immunohistochemistry. H and E sections will be obtained to examine morphology, and TUNNEL analysis will be performed to determine at what point cells undergo apoptosis following Bcl-x$_S$ expression.

<u>Task 3.3</u>: Determine the effect of Bcl-x$_S$ on the growth of breast tumors *in vivo*

Once more thorough analysis of the WAP-Bcl-x$_S$-HA transgenic mice is complete, we plan to analyze the effect of Bcl-x$_S$ on the growth of breast tumors *in vivo*. We plan to mate these mice to transgenic mice that have an increased incidence of breast tumors due to over-expression of an oncogene, ideally one that is also under the control of the WAP promoter. Candidates for such tumor producing mice include WAP-Bcl-2/Myc mice, WAP-TGFα mice, WAP-IGF-1 mice and WAP mutant p53 mice.

CONCLUSIONS

The studies that we have performed in the past year are important in that they have provided information regarding the mechanism by which the Bcl-x$_S$ kills tumor cells. Bcl-x$_S$ physically associated with Bcl-x$_L$ and Bcl-2 in cancer cells although the binding to Bcl-x$_L$ was stronger than with Bcl-2. Mutational analysis has revealed that the BH3 domain of Bcl-x$_S$ is critical for Bcl-x$_L$ binding and induction of apoptosis. Particularly revealing was the interaction of Bcl-x$_L$ and Bcl-x$_S$ with Apaf-1, a mammalian counterpart of nematode CED-4. These results suggest that Bcl-x$_S$ promotes apoptosis by antagonizing Bcl-x$_L$ through Apaf-1.

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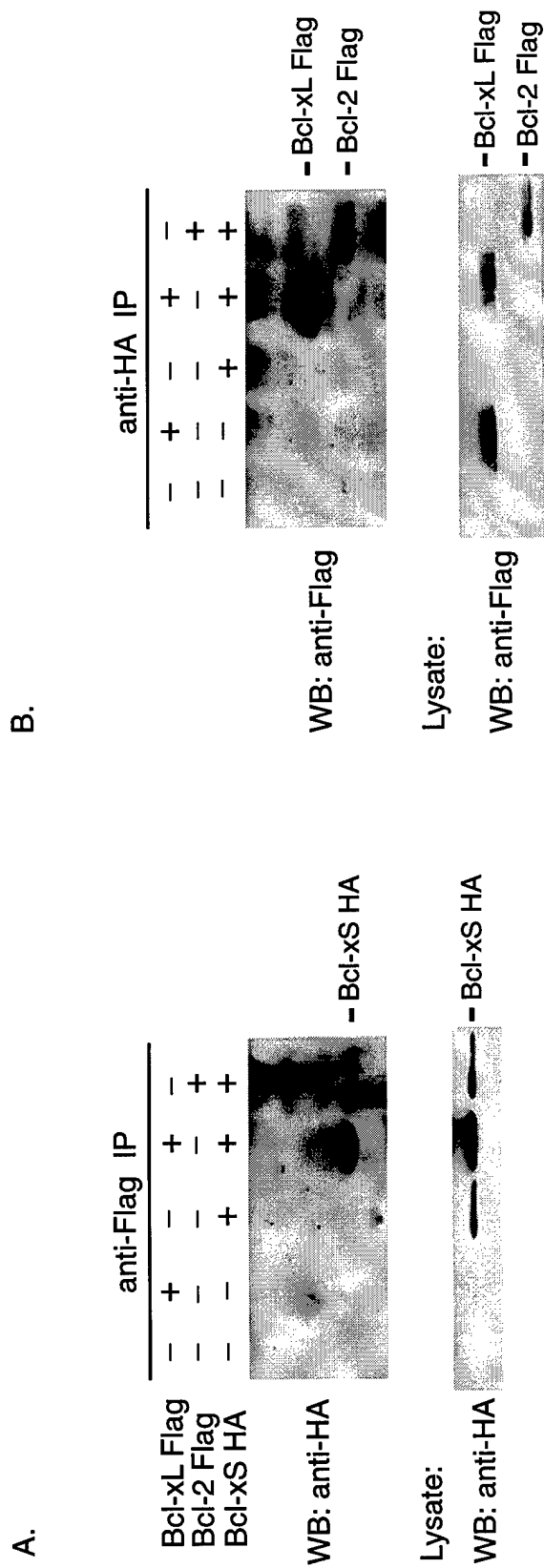
APPENDIX

Figure 1

A.

anti-Flag IP

Bcl-xL Flag	−	+	−	−	+	−
Bcl-2 Flag	−	−	+	−	−	+
Bcl-xS HA	−	−	−	+	+	+

WB: anti-HA

— Bcl-xS HA

Lysate:

WB: anti-HA

— Bcl-xS HA

B.

anti-HA IP

−	+	−	−	+	−
−	−	+	−	−	+
−	−	−	+	+	+

WB: anti-Flag

— Bcl-xL Flag
— Bcl-2 Flag

Lysate:

WB: anti-Flag

— Bcl-xL Flag
— Bcl-2 Flag

Figure 1 Bcl-xs-HA associates with both Bcl-XL-Flag and Bcl-2-Flag in cancer cells

Cancer cells were transiently transfected with Bcl-xs-HA and Bcl-xL-Flag or Bcl-2-Flag.
Anti-Flag (A) or Anti-HA (B) complexes were immunoprecipitated and immunoblotted with the
reciprocal antibody. The lower panels are immunoblots of transfected cell lysates.
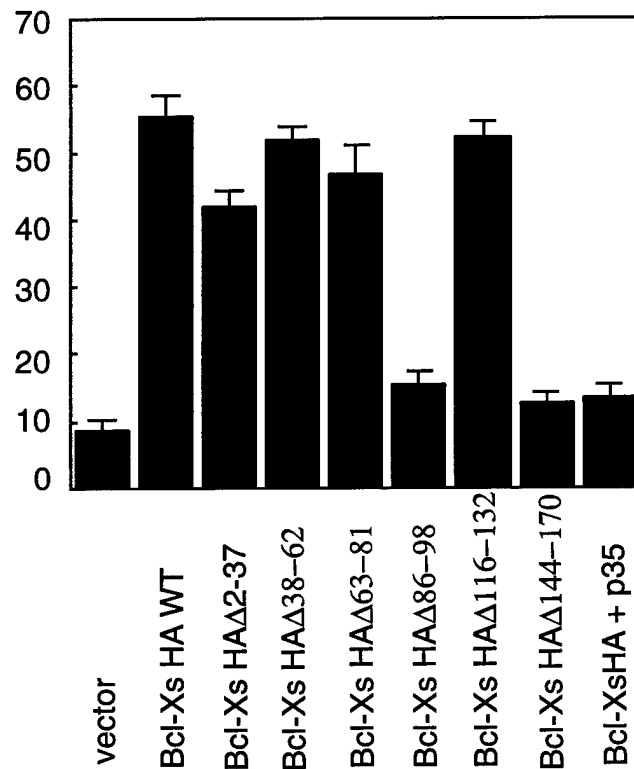
Figure 2

A.
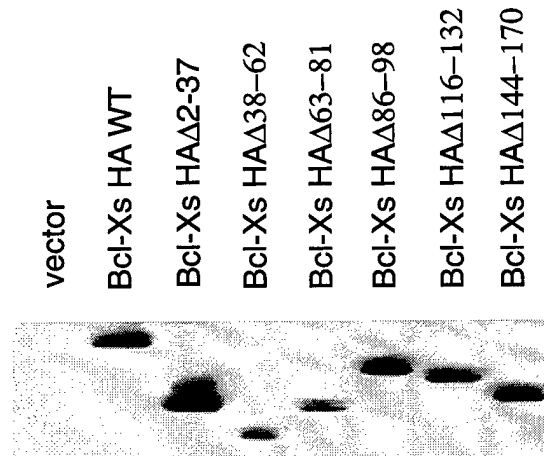
B.

Figure 2 Deletion Analysis of Bcl-xS

A. Anti-HA Western Blot of HA-Bcl-xs mutants transiently expressed in cancer cells.
B. Transient transfection assay to measure Bcl-xs killing of cancer cells. Plasmids were co-transfected with pCMV Bgal; 18 hrs. post-transfection, cells were stained with X-gal and % blue cells with apoptotic morphology was measured.
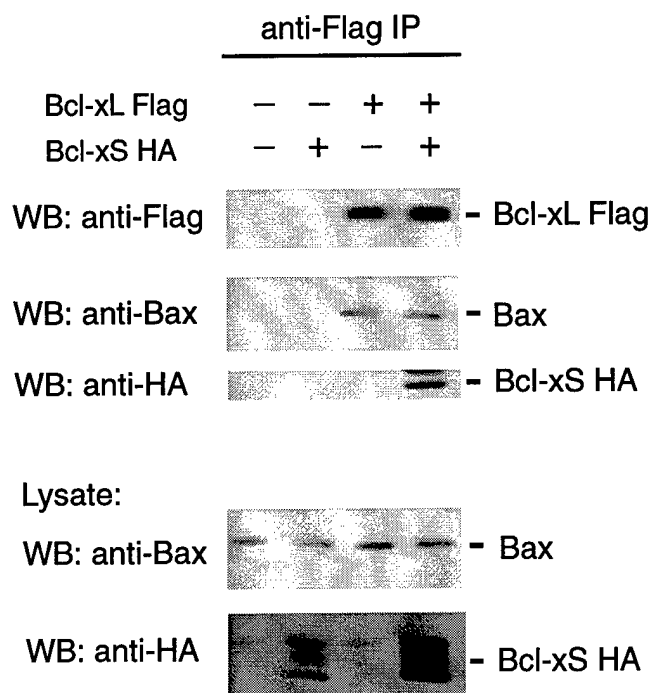
Figure 3

Figure 3 Bcl-xs fails to disrupt Bax/Bcl-XL complexes

Cancer cells were transiently transfected with Bcl-xs-HA and/or Bcl-xL-Flag.
Endogenous Bax/Bcl-xl complexes were immunoprecipitated with anti-Flag
and immunoblotted with either anti-Bax, anti-HA or anti-Flag. The lower panels
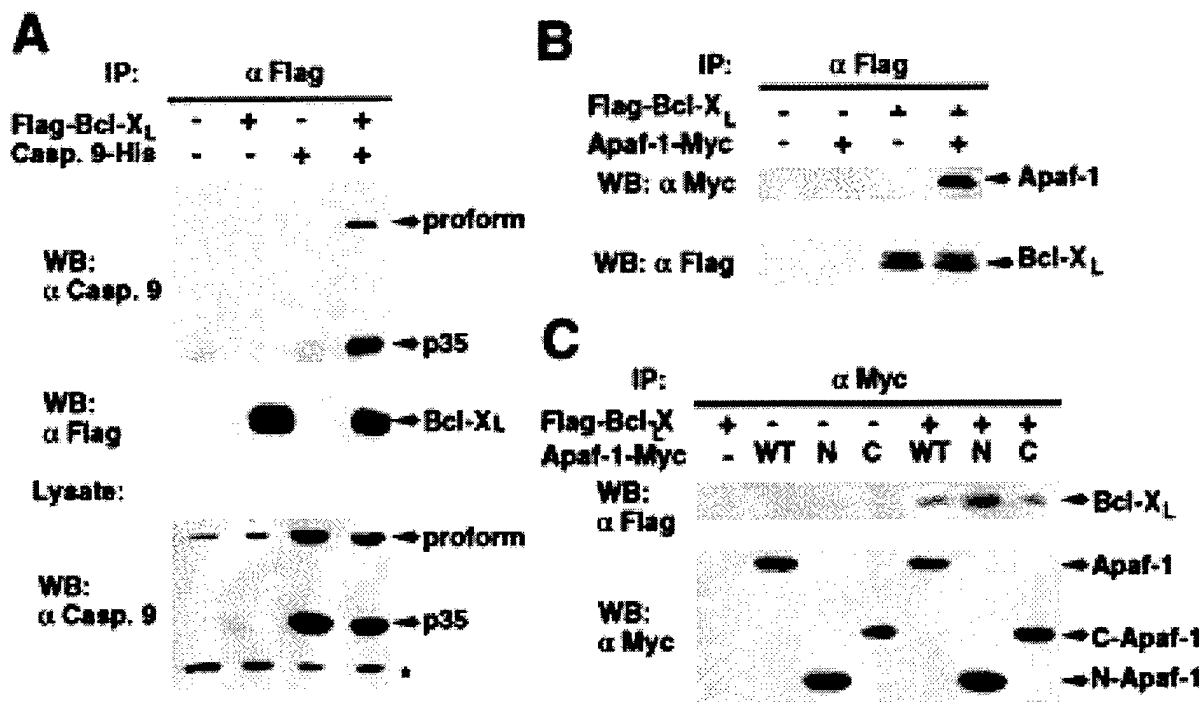are anti-Bax and anti-HA immunoblots of transfected cell lysates.

A

IP: α Flag

Flag-Bcl-X_L   - + - +
Casp. 9-His   - - + +

→ proform

WB:
α Casp. 9

→ p35

WB:
α Flag   →Bcl-X_L

Lysate:

→ proform

WB:
α Casp. 9   →p35
*

B

IP: α Flag

Flag-Bcl-X_L   - - + +
Apaf-1-Myc   - + - +

WB: α Myc   → Apaf-1

WB: α Flag   →Bcl-X_L

C

IP: α Myc

Flag-Bcl_LX   + - - - + + +
Apaf-1-Myc   - WT N C WT N C

WB: →Bcl-X_L
α Flag

→Apaf-1

WB: →C-Apaf-1
α Myc
    →N-Apaf-1

FIGURE 4. Bcl-x_L interacts with caspase-9 and Apaf-1. (A) Bcl-x_L interacts with caspase-9. (Upper) Western blot analysis of immunoprecipitated Bcl-x_L and coimmunoprecipitated pro-caspase-9 and processed form (p35). (Lower) The expression of caspase-9 in total lysate. (B and C) Bcl-x_L interacts with Apaf-1. 293T cells were transfected with indicated plasmids and the lysates immunoprecipitated with anti-Flag (B) or anti-Myc (C) antibody. WT, full length Apaf-1-Myc; N, N-terminal Apaf-1-Myc; C, C-terminal Apaf-1-Myc. Panels show Western blot analysis of coimmunoprecipitated Apaf-1 and Bcl-x_L proteins. Asterisk indicates nonspecific band.
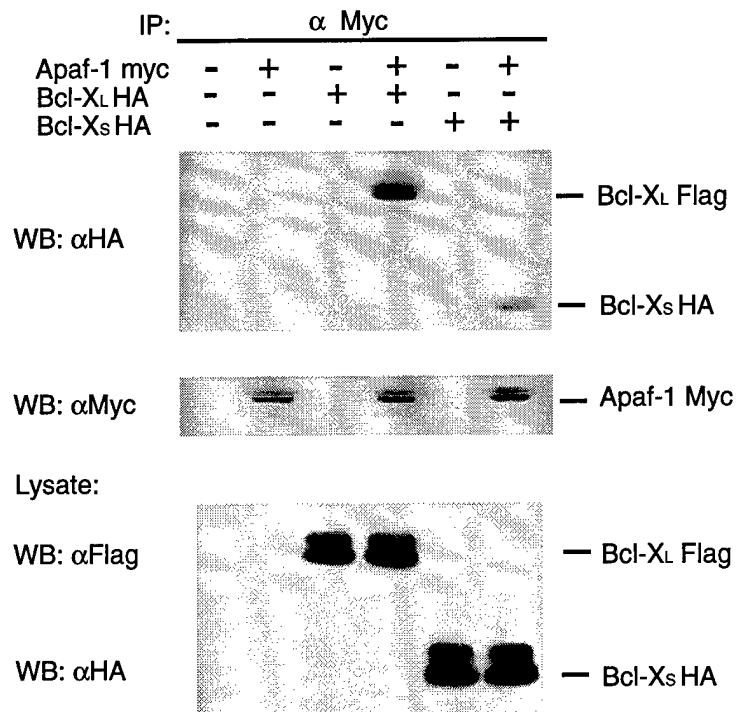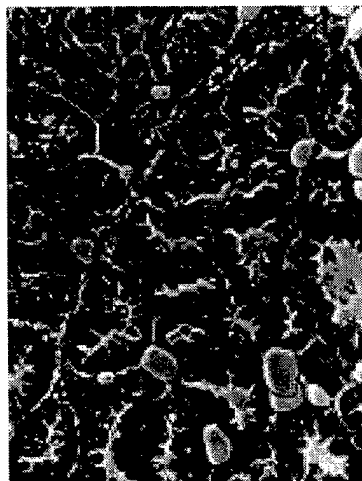
Figure 5

Figure 5 Bcl-xs-HA and Bcl-XL-Flag co-precipitate with Apaf-1 Myc

Cancer cells were transiently transfected with Apaf-1 Myc and Bcl-xs-HA
or Bcl-xL-Flag. Apaf-1 complexes were immunoprecipitated with anti-Myc
antibody and immunoblotted with anti-Flag, anti-HA, or anti-Myc. Lower
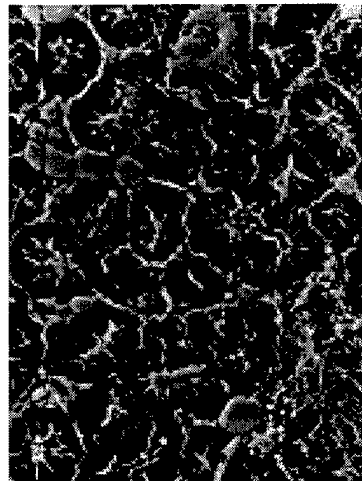panels are immunoblots of transfected cell lysates.

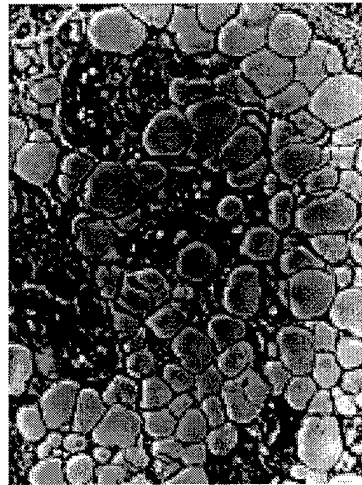Figure 6

WB: anti-bcl-x

A.

Fig. X Wap-Bcl-xS transgenic mouse

A. Anti-Bcl-x western blot of mammary gland tissue harvested at day L10 of lactation. Lines 1-6 are positive for Bcl-xS by PCR. Of these, only line 3 expressed Bcl-xS protein. Pos. control lysate is from a cell line stably over-expressing Bcl-xL-Flag and Bcl-xS-HA. B-D. H and E stained sections of L10 mammary gland from: B: negative control littermate (first pregnancy), C: Wap-Bcl-xS mouse-line 3 (first pregnancy), and D: same Wap-Bcl-xS mouse (second pregnancy.)